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Contact Killing of Gram-Positive and Gram-Negative Bacteria on PDMS Provided with Immobilized Hyperbranched Antibacterial Coatings

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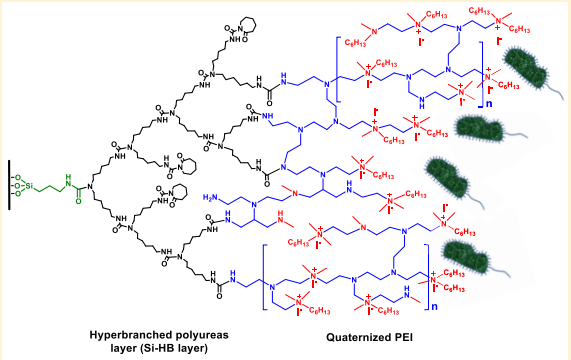
Supporting Information

ABSTRACT: Here we describe in detail the preparation and application of antibacterial coatings on PDMS (poly(dimethylsiloxane)) and the contact-killing properties with 10 bacterial strains. Our aim was to develop a generally applicable coating to prevent biomaterial acquired infections, which is the major mode of failure of biomedical implants. In the first step, the surface was provided with a hydrophobic hyperbranched coating resin that was covalently attached to PDMS, mediated by an appropriate coupling agent. The coupling agent contained a siloxane group that reacts covalently with the silanol groups of air-plasma-treated PDMS and a blocked isocyanate enabling covalent coupling with the amino groups of the hyperbranched coating resins. The coating resins were functionalized with a polyethylenimine and subsequently quaternized with bromohexane and iodomethane. The coatings were highly effective against Gram-positive bacteria (five strains) and sufficiently active against Gram-negative bacteria (five stains). The killing effect on the latter group was strongly enhanced by adding a permeabilizer (EDTA). The biocidal efficacy was not influenced by the presence of (saliva) proteins.



INTRODUCTION

Biomaterial based implants and devices are widely applied to restore human function and shape once beyond natural repair, such as after trauma, invasive oncological surgery, or simply wear. Biomaterials can also be used for the temporary support of function during hospitalization, as construction materials for biomedical implants, or in the administration of medication. Their use is increasing because of the increased life expectancy worldwide and the growing demands of patients for "healthy aging" with full body functioning from birth until death. However, biomaterial-associated infections caused by implants are still a major drawback.[1−4]

Biomedical implants are composed of metal, ceramic, or polymeric materials depending on the specific requirements, in particular, mechanical load and biostability/biodegradability. The development of biomedical devices is a complex, time-consuming, and expensive process. This might be the reason that during these developments little or no attention is paid to postoperative infections. Nevertheless, it remains remarkable when realizing that in spite of all cleaning procedures performed in operating theaters, devices are always contaminated with (pathogenic) bacteria.[5] In most cases, the immune system and/or administered antibiotics can take care of clearance from the body, but in roughly 5% of all cases, infections will prevail and cause severe illness or even mortality.[6]

The poor efficacy of antimicrobial treatments of biomaterial-associated infections together with the rapid emergence of new resistant microbial strains has stimulated the development of a variety of coatings that either locally release antimicrobials, are nonadhesive to microorganisms, or kill adhering organisms upon contact. When antibiotics are used, they release, after a burst release, traces thereof for a longer time, which can create resistance. Contact-killing coatings are therefore considered to be highly promising because they are not exhausted and do not release biocides into body fluids. Most of the contact-killing coatings comprise quaternary ammonium compounds (QACs), provided with a hydrophobic alkyl chain, mimicking natural antibacterial peptides.[7] When the cationic charge density is above a certain threshold (>10^{15} N^+ atoms/cm^2), it

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kills bacteria effectively upon contact.[8] Although the exact mechanisms of the bacterial contact-killing process by QACs is still under debate, membrane disruption is essential in all proposed mechanisms.[9]

Soluble QACs are well known to be potent biocides and have been used for many decades in disinfectants and cosmetic products[10] without building up resistance,[11] but they are still not totally safe for human cells.[12] Antibacterial coatings comprising leachable QACs (e.g., chlorohexidine) will give an undesired contamination of body fluids and will, in addition, be exhausted in due time.[13,14] In the past decade, much attention has therefore been paid to preventing leaching by immobilizing biocidal quaternary ammonium compounds.[15,16] In one approach, living radical polymerization is started from grafted initiators. The monomers comprised amino groups that were either alkylated afterward or contained quaternary ammonium moieties.[17−19] Other researchers have grafted the antibacterial polymers, peptides, or quaternized polyamines directly onto reactive groups on the surface.[20,21] However, in all of these cases only nanometer-thick organic coatings have been obtained, which are vulnerable for hydrolysis and damage because of high mechanical forces during surgery.

The aim of this study was to investigate in detail the preparation of robust hyperbranched antibacterial coatings on PDMS (poly(dimethylsiloxane)) and to show their ability to kill a series of 10 different bacterial strains on contact. To achieve firm adhesion on the substrate an appropriate coupling agent was prepared. In particular on PDMS, which is frequently used in biomaterial implants and devices, an appropriate coupling agent was indispensable as the physical interaction of coatings with PDMS is by far too low to achieve firm attachment. Hydrophobic hyperbranched coating resins were covalently fixed on the coupling agent and provided ammonium moieties with covalently tethered quaternary biocidal agents. The antibacterial contact-killing properties of QAC-coated PDMS sheets were evaluated for five Gram-positive and five Gram-negative bacterial strains. Temporary PDMS implants and devices, such as catheters and voice prostheses, are possible targets for this study. They are a relatively fast, applicable downward clinical translation of antimicrobial temporary implants, faster than in applications of permanent implants and devices.

EXPERIMENTAL SECTION

Materials. PDMS sheets were kindly obtained from Atos Medical, Sweden. Bishexamethylene triamine, (3-aminopropyl) triethoxysilane, polyethylenimine (750 kDa, 50 wt % in water), iodomethane, 2-methyl-2-butanol, 1-bromohexane 1,8-bis(dimethylamino)-naphthalene, and EDTA were purchased from Sigma-Aldrich. Methanol, ethanol, *n*-hexane, toluene dimethylformamide were obtained from Lab-Scan. Carbonyl biscaprolactam (CBC; > 99%) was provided by Actu-all, Oss, The Netherlands. All chemicals were used as received.

Synthesis. Syntheses of AB_2 monomers (**1**), the corresponding hyperbranched polymers (**2**), and the coupling agent (**3**) were described previously.[25,26]

PDMS Extraction. PDMS sheets were cut into small pieces (2 × 2.5 cm^2 and 0.05 cm thick) and immersed in toluene for 24 h at room temperature while stirring to extract the low-molecular-weight compounds. PDMS slides were next dried overnight in an oven at 50 °C. The percentage extraction was determined by dividing the difference between the weight of PDMS before and after extraction by the original weight of PDMS.

Application of Hyperbranched Polymer Coatings (PDMS-HB) (2). PDMS pieces (2 × 2.5 cm^2) were sonicated for 10 min at RT (room temperature) in ethanol and dried. Next, samples were placed in air plasma equipment (Femto system from Diener-electronic, Germany) at 100 W for 1 to 2 min (in 1.7 × 10^{-1}mbar air). The effectiveness of the plasma treatment was monitored by water contact angle measurements. The obtained hydrophilic PDMS pieces were immersed in a 3 v/v% solution of the coupling agent (**3**) in absolute ethanol for 10 min at RT and then placed in a vacuum oven and held at 110 °C for 2 h under vacuum. The unreacted coupling agent was removed by washing PDMS pieces in ethanol for 20 min in a sonic bath at RT and was dried under vacuum and stored under nitrogen. PDMS slides were submerged in a solution of hyperbranched polymer (**2**) (5 wt % in ethanol) and subsequently spin coated (2000 rpm, 60 s). Fixation on the coupling agent as well as a continued polymerization of hyperbranched polymer (**2**) on the surface was carried out while holding the sample at 145 °C for 2 h under a flow of nitrogen after evaporation of the ethanol under vacuum. Non-anchored polymers were removed by sonication in absolute ethanol for 20 min at RT and extracted in 200 mL of DMF at 115 °C overnight. Next, PDMS pieces were sonicated in absolute ethanol for 20 min at RT and dried and stored under nitrogen.

Caprolactam-Blocked Isocyanatopropyl Triethoxysilane (3, Coupling Agent).[26] In a three-necked flask provided with a reflux condenser, carbonyl biscaprolactam (11.34 g, 45 mmol) and (3-aminopropyl) triethoxysilane (9.95 g, 45 mmol) were dissolved in 40 mL of toluene. The reaction was carried out under nitrogen at 80 °C for 6 h. Toluene was distilled under reduced pressure. The obtained coupling agent was stored under nitrogen.

Modification of a Hyperbranched Coating with Polyethylenimines (4). A solution of polyethylenimine (PEI) in water (50 wt %) was freeze-dried overnight (M_w = 750 kDa), and the residue was dissolved in methanol at 20 wt % concentrations. The PEI solution (200 μL) was dropped onto **2** and spin coated (2000 rpm, 60 s). The anchoring reactions were carried out on an aluminum plate at 125 °C for 24 h under nitrogen. Unreacted PEI was removed with methanol using an ultrasonic bath for 45 min at RT and dried under nitrogen. Yellow coatings were obtained after the coupling reaction of PEI.

Alkylation of a PEI-Functionalized Hyperbranched Coating (PDMS-HB-PEI+) (5). In a round-bottomed flask provided with a reflux condenser, coatings comprising tethered PEI were immersed in 75 mL of 1-bromohexane and heated under nitrogen at 90 °C overnight. Next, 1.07 g of a proton sponge (1,8-bis(dimethylamino)-naphthalene) in 25 mL of *tert*-amyl alcohol was added. The reaction was continued for another 3 h at 90 °C. Afterward, the coatings were sonicated three times in methanol for 20 min at RT and dried under nitrogen.

A second alkylation step was performed in a round-bottomed flask fitted with a reflux condenser. Samples were immersed in a solution of 10 mL of iodomethane in 75 mL of *tert*-amyl alcohol. The alkylation reaction was carried out at 42 °C overnight; the samples were subsequently sonicated in methanol for 20 min at RT, followed by extraction in methanol at 65 °C for 1 day and another sonication in methanol for 20 min at RT. The obtained coatings were dried and stored under nitrogen.

ATR-FTIR. Infrared spectra were recorded on a PerkinElmer "Spectrum 400" FT-IR spectrometer using a UATR (universal attenuated total reflection) attachment and a liquid-nitrogen-cooled mercury cadmium telluride detector.

X-ray Photoelectron Spectroscopy. XPS was performed using an S-Probe spectrometer (SurfaceScience Instruments, Mountain View, CA, USA) equipped with an aluminum anode (10 kV, 22 mA) and a quartz monochromator on 2.6 × 2.6 cm^2 glass slides. The direction of the photoelectron collection angle was 55° with the normal to the sample, implying the measurement of composition over the outermost 10 nm of the coating (i.e., the approximate electron mean free path length in polymers). The electron flood gun was set at 10 eV. A survey scan over a binding energy range of 1100 eV was made with a 1000 × 250 μm^2 spot and a pass energy of 150 eV. Binding energies were determined by setting the binding energy of the C 1s binding energy peak (carbon bound to carbon) to 284.8 eV. Detailed scans of the N 1s binding energy peaks over a binding energy

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range of 20 eV were made using a pass energy of 50 eV. The N 1s peak was subsequently decomposed in two fractions at 399.3 and 401.3 eV. The occurrence of a peak at 401.3 eV was interpreted as being due to alkylated nitrogen species and was expressed in atom % by multiplication of the peak fraction by the total atom % nitrogen.

AFM Measurements. A BioScope Catalyst AFM with ScanAsyst (Veeco, Camarillo, CA, USA) was used for imaging. Deflection images were taken over various surface areas. The scans were made in contact mode under the lowest possible applied force (1 to 2 nN) at a scan rate of 1 Hz using DNP probes from Veeco (Woodbury, USA).

Dektak Stylus Profiler. The thickness of HBP coatings was measured with a Dektak 6 M stylus profiler. The radius of the stylus is 2.5 μm with a length of 800 μm. The duration time is 20 s with a force of 3 mg. The measurement range is 65 kÅ with a resolution of 0.133 μm/sample. The scan mode is a standard scan with a profiler of hills and valleys. Several scratches were made on the layer with a razor blade and measured through the aforementioned procedure.

Fluorescein Method. The cationic charge density of the coatings was determined using fluorescein staining. To this end, coated slides were immersed at RT in 15 mL of 1 wt % fluorescein (disodium salt) solution in demineralized water for 10 min and washed four times with 50 mL of water, followed by sonication in 50 mL of water for 5 min at RT to remove any dye not complexed with cationic charges. Next, the samples were placed in 10 mL of a 0.1 wt % cetyltrimethylammonium chloride solution in demineralized water and sonicated for 10 min at RT to desorb complexed fluorescein dye. Subsequently, 10 v/v % of 100 mM phosphate buffer, pH 8, was added to a total volume of 11 mL, and UV/vis measurements (Spectra max M2 UV/vis spectrophotometer) was carried out at 501 nm to yield the concentration of fluorescein dye in the extraction solution [dye] in M according to

$$[\text{dye}] = \text{Abs } 501/\varepsilon_{501}\text{L} \quad (1)$$

in which Abs 501 is the UV absorption at 501 nm, ε_{501} is the extinction coefficient (77 mM^{-1} cm^{-1} for fluorescein), and L is the length of a polystyrene cuvette (1 cm) traversed by the UV-light beam. Next, the cationic charge density per cm^2 glass slide was calculated using eq 2[8]

$$\text{charge density} = [\text{dye}]VN/A \quad (2)$$

in which V is the volume of the extraction solution (11 mL), N is Avogadro's number (6.023×10^{23}), and A is the surface area of the slide (5.00 cm^2).

Antibacterial Measurements. Gram-positive (*Staphylococcus epidermidis* ATCC 12228, *S. epidermidis* 3018, *Staphylococcus aureus* ATCC 12600, *Streptococcus salivarius* GB 24/9, *Rothia dentocariosa* GBJ 52/2B) and Gram-negative (*Escherichia coli* ATCC 15597, *E. coli* Hu 734, *E. coli* 1110, *Proteus mirabilis* 28cii, *P. mirabilis* 296) bacteria were used in this study. The strains were first streaked on blood agar plates from frozen stock solutions (7 v/v % DMSO) and grown overnight at 37 °C. One colony was inoculated in 10 mL of tryptone soya broth (TSB, Oxoid, Basingstoke, U.K.) for the staphylococcal strains and in brain−heart infusion broth (BHI, Oxoid) for *E. coli* and was incubated at 37 °C for 24 h. These cultures were used to inoculate a main culture of 200 mL of TSB or BHI, which was incubated for 16 h at 37 °C. Bacteria were harvested by centrifugation for 5 min at 6500*g* and 10 °C and subsequently washed two times with 10 mM potassium-phosphate buffer at pH 7.0. Bacterial challenge concentrations of 10^2, 10^3, and 10^4 colony-forming units (CFU)/mL were used. The contact killing of the coatings was investigated by employing a Petrifilm Aerobic Count plate system (3 M Microbiology, St. Paul, MN, USA) as described before.[8] A bacterial suspension (40 μL) was placed on bare as well as on functionalized PDMS slides (2.5 × 2.5 cm^2) and placed in the Petrifilm system before the Petrifilm system was closed. The bacterial suspension spread over the entire surface area of the samples, enabling the calculation of the bacterial challenge per cm^2 from the dimensions of the samples and the bacterial concentration in suspension. Petrifilms were incubated at 37 °C for 48 h, after which the numbers of CFUs were counted. As a control, 40 μL of the bacterial suspension was inoculated in a Petrifilm system without a sample.

In the case of Gram-negative bacteria, EDTA was additionally added to the bacterial suspensions at a concentration of 0.1 mM. After the top lid was placed on the coated samples, the Petrifilms were incubated at 37 °C for 48 h. A bacterial suspension without a sample in between the films (10 μL) was used as a control. After the incubation, CFUs were counted and the CFUs per cm^2 was calculated.

Some experiments were performed in the presence of salivary conditioning films to determine if the coating was still active when covered with a conditioning film. After stimulation by chewing Parafilm, human whole saliva from 20 healthy volunteers of both sexes was collected into ice-cooled beakers, pooled, centrifuged, dialyzed, and lyophilized for storage. Prior to lyophilization, phenylmethylsulfonylfluoride (final concentration 1 mM) was added as a protease inhibitor in order to reduce protein breakdown and preserve high-molecular-weight mucins. For experiments, lyophilized saliva was dissolved at a concentration of 1.5 mg/mL in buffer (2 mM potassium phosphate, 50 mM potassium chloride, and 1 mM calcium chloride at pH 6.8). All volunteers gave their informed consent to saliva donation in accordance with the rules set by the Medical Ethical Committee at the University Medical Center Groningen, Groningen, The Netherlands (letter 06-02-20090. Coated and uncoated PDMS pieces were placed in reconstituted human whole saliva for 16 h at room temperature in order to form a SCF. After adsorption, PDMS pieces were rinsed three times with sterile demineralized water to remove excess of saliva and used in a Petrifilm test as described above.

RESULTS AND DISCUSSION

Biomaterials are favored zones to be colonized by bacteria, who protect themselves subsequently against the immune system and administered antibiotics by an extracellular polymeric matrix, a biofilm. To protect patients from infections by preventing biofilm formation, bacteria should be killed as soon as they contact implant surfaces. It was not unreasonable to assume that application of immobilized biocidal coatings on implants could be a general methodology to prevent biomaterial acquired infections. In contrast, coatings with leachable biocides (e.g., silver) or antibiotics (such as gentamicin), as have been reported, are not favored because of the contamination of bodily fluids, their exhaustion in time, and the likelihood of creating resistance. Covalent attachment of biocidal compounds on implants could circumvent these problems.

PDMS. Although poly(dimethylsiloxane) (PDMS) is a highly cross-linked polymer, it will, as will all thermosets, contain a fraction of low-molecular-weight compounds that have a tendency to migrate to the surface. This fraction may form a weak interlayer between the substrate and a coating, and it is therefore highly recommended to remove this fraction. Extracted with toluene revealed the presence of about 2 wt % of low-molecular-weight compounds, making the PDMS sheets suitable for allying immobilized coatings.

The adhesion forces of coatings on pristine PDMS were too weak to generate any adherence. Suitable anchoring groups (≡SiOH, silanol) were created by air−plasma treatment. The formation of silanol groups was monitored by a strong decrease in the water contact angle from 115 to less than 20°. However, if the surface energy is higher than in the bulk, then the polar (silanol) groups will migrate into the bulk of the material, making the surface hydrophobic again.[23] It was therefore necessary to couple the coupling agent directly to the silanol groups after the plasma treatment.

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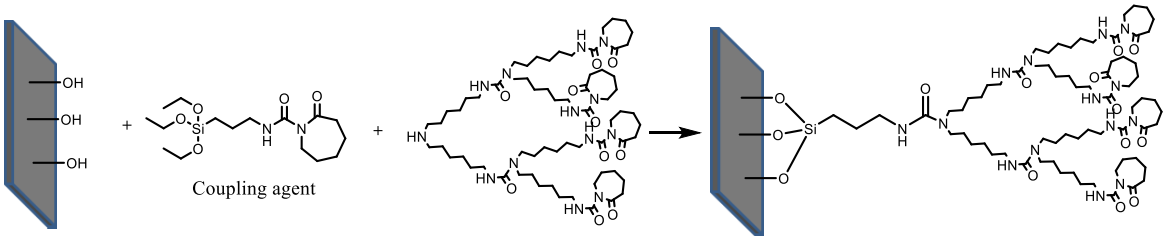
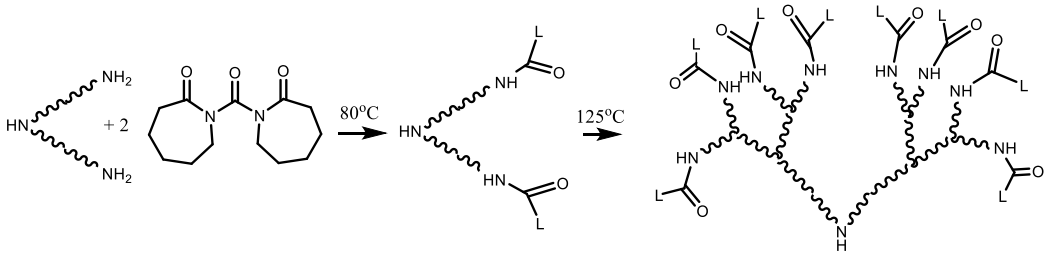
Scheme 1. Schematic Representation of the Coupling of Silanol Groups and the Amino Group of the Hyperbranched Polymer, Mediated by the Coupling Agent



Scheme 2. Schematic Overview of the Synthesis of AB_2 Monomers and the Corresponding Hyperbranched Polymer Starting from Hexamethylene Triamine and CBC (L = Caprolactam)



Coupling Agent. An appropriate coupling agent (CA) should be provided with two different functional groups: one to react with silanol groups on the surface and a second one to react with the coating resin. The two reactive groups should be chosen in such a way that they do not react with each other, while the second one should not react with silanol groups on the surface. γ-Aminopropyltriethoxysilane (APTES) has been used for decades as a coupling agent, introducing an amino group on silanol comprising surfaces as an anchor moiety.[24] However, an amino group was not able to react with our hyperbranched polymers (see below). Therefore, a blocked isocyanate (BI) derivative of APTES was prepared.[25] This coupling agent was obtained by reacting APTES with carbonyl biscaprolactam, forming in one step the corresponding BI in quantitative yield.[26] Advantage was taken of the high selectivity of BIs to react with amino and not with silanol groups under the applied conditions (Scheme 1).

After the coupling agent was applied by a spin-coating procedure, the samples were heated to 110 °C for 2 h to create a covalent bond between the coupling agent and silanol groups while retaining the BIs. ATR-FTIR revealed the presence of a thin layer of the coupling agent, as evidenced by the acyclic carbonyl peak at 1650 cm^{-1}. The presence of a caprolactam blocking group of the BI was proven by the carbonyl peak at 1705 cm^{-1}. The XPS data revealed the presence of carbon atoms with electron binding energies of around 288 eV (286.4, 287.4, 288.0, and 290.0 eV) and nitrogen atoms (400.0 eV), of which the latter was not present on the pristine PDMS surface. Additional evidence was obtained from water contact angle measurement, showing an increase from less than 20 to 75 ± 3° upon applying the coupling agent.

It was not possible to prove directly that the blocked isocyanates of the coupling agents would react with the secondary amino group of the hyperbranched polymer due to the masking effect of the polymer layer. Therefore, a model reaction was conducted with the immobilized CA and dibutylamine. After heating a spin-coated layer of dibutylamine on top of the applied CA, the carbonyl peak in the ATR-IR spectrum at 1705 and the CH_2 scissor vibration at 1440 cm^{-1} of the caprolactam rings disappeared. In the XPS spectrum, the peak at 285.8 eV, due to aliphatic hydrocarbons, became dominant, and the carbon peaks of C−N at 287.5 eV and of C═O at 289.6 eV were reduced. It was reasonable to expect that the secondary amine of the AB_2 monomers would react in the same way as with dibutyl amine.

Hyperbranched Polymer. AB_2 monomers for the preparation of hyperbranched polymers (HBPs) were obtained in a one-step reaction from commercially available bis(hexamethylene) triamine and carbonyl biscaprolactam (CBC, Scheme 2).[26]

Because of the extremely high selectivity of CBC, the primary amines reacted exclusively, quantitatively yielding AB_2 monomers with one secondary amino group and two BIs. After spin coating the AB_2 monomers on the surfaces covered with the coupling agent, the polymerization started upon heating at 145 °C. The secondary amino groups were able to react with the blocked isocyanates, forming hyperbranched polyurea (HBPs) coatings. Polyurea are very well suited for biomedical applications because of their good biocompatibility.[28,29] Initially, the AB_2 monomers were polymerized as such on the surface. However, it appeared to be challenging to prepare homogeneous coatings due to some dewetting. It was found that a 5 wt % solution of the HBPs with a number-average molecular weight of 1500 Da (according to ^1H NMR) gave the desired solution viscosity for preparing homogeneous coatings. Obviously, such low-molecular-weight polycondensates still contain a substantial quantity of AB_2 monomers, but there was no need to remove them because they also polymerize at 145 °C on the surface, together with the oligomers. During the bulk polycondensation process on the surface, the molecular weight of the HBP increased, and as a consequence, the thickness of the immobilized coating increased as well, depending on the quantity of applied AB_2 monomers/HBP, polymerization temperature, and time. The thickness could be controlled from nanometers up to micrometers because of the living character of the polycondensation reaction. Hyperbranched polymers have the advantage of having many end groups, so even when some end groups became dead ends the

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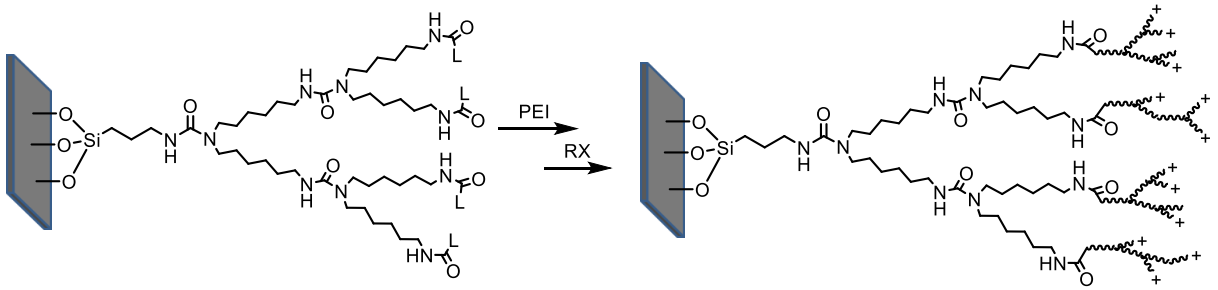
Scheme 3. Schematic Representation of the Modification of a Hyperbranched Polymer (HBP) with Polyethylenimine (PEI) That Is Subsequently Quaternized by Alkyl Halides (RX), with L = Caprolactam



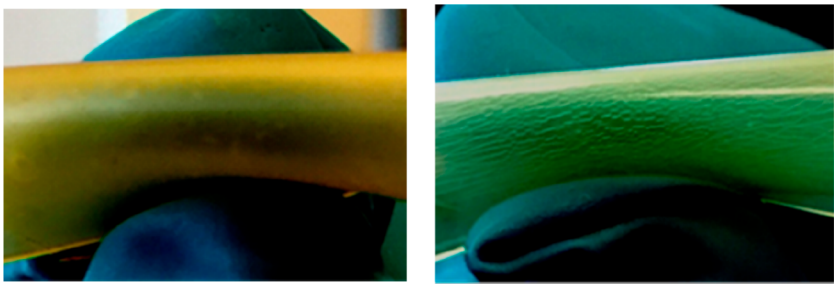


Figure 1. QAC coatings on PDMS after cross-linking of the immobilized HBPs with a polyamine (PEI). Curing for 3 h at 125 °C yielded a flexible coating (left panel), and curing for 16 h created a highly cross-linked coating that wrinkles upon bending (right panel).

polymerization was still able to continue.[22] After the polymerization of the first layer, an additional layer could be applied by adding a new quantity of monomers or oligomers and heating it again. Thickness and thickness increase measurements before and after adding the second amount of AB_2 monomer/oligomer were carried out on glass slides instead of on PDMS because a hard surface gave more reliable results when making scratches with a razor blade. The thicknesses depended on the concentration of the resin. The layer thickness of the resin spin-coated from a 5 wt % ethanol solution was 0.207 ± 0.03 μm while being 0.988 ± 0.06 μm from a 20 wt % ethanol solution. By repeating the spin-coating process with the 20 wt % resin in ethanol on the first layer, an increase from 0.988 ± 0.06 to 1.407 ± 0.05 μm was found. This demonstrates that by repeating the same spin-coating procedure thicker coatings can be prepared.[30] The non-attached polymer was removed by an extraction with DMF at 115 °C overnight. These were severe conditions to guaranty that there were absolutely no leachables.

Functionalization. The immobilized HBPs comprised numerous blocked isocyanate groups at the surface. As expected, these coated samples were storage-stable and could be stored for months because it is well known that polyurea and blocked isocyanates are not reactive or sensitive to moisture at ambient temperature. This enabled the introduction of a variety of functional compounds on the surface upon heating if they were provided with −OH or −NH groups. Modification with monomethoxy polyethylene glycol resulted in a decrease in the water contact angle from 75 ± 3 to 40 ± 3°, yielding hydrophilic surfaces. Modification with perfluorodecanol increased the water contact angle to 104 ± 4°, resulting in a hydrophobic surface.[27] Although hydrophilic and hydrophobic surfaces are often reported to reduce bacterial adhesion, they only delay adhesion but cannot prevent the formation of biofilms.[31] In contrast, immobilized QACs kill bacteria as soon as they contact the surface, provided that the charge density is above a certain threshold ($>10^{15}N^+/cm^2$).[8] To obtain such a high charge density, a high-molecular-weight polyamine (polyethylenimine, PEI, M_w = 750 kDa) was tethered through a reaction with the BIs of the HBP (Scheme 3).

ATR-FTIR showed the carbonyl peak at 1668 cm^{-1} (ureido) and amino groups at 3364 cm^{-1}, while the peak at 1705 cm^{-1} (caprolactam) vanished.

PEI was not only of importance in introducing numerous positive charges but offered here in addition the opportunity to cross-link the hyperbranched coating. PDMS is flexible and needs flexible coatings and thus a low cross-linking density, while for metals a high cross-link density was preferred. The cross-linking density depended on the amount of PEI and the reaction temperature and time. It was found that after a heating time of 3 h at 125 °C the coating was still flexible and could be bent without damage. After prolonged heating (16 h), the coating became stiff, which was not applicable any more for PDMS (Figure 1). An important indication of the mechanical properties of coatings is the acetone double-rub test[32] in which a paper tissue soaked in acetone is rubbed up and down until visible damage occurs. Here, the coating could withstand 100 acetone double rubs, the first indication of rather good wear resistance.

Alkylation. Initially, the alkylation step of PEI with alkyl halides was conducted in a suspension of KOH as an acid scavenger (HBr and HI), as described in the literature.[33] However, it appeared that this procedure was too aggressive for PDMS. Delamination of the coating ("peeling off") was observed (Figure S1). We found that with 1,8-bis(dimethylamino)naphthalene (a proton sponge) as a base, no visible damage to the coating could be observed. The AFM roughness of the surface was 2.3 ± 0.3 μm when KOH was used as base and 0.8 ± 0.2 μm in the presence of the proton sponge. It should be emphasized that bacteria adhere less on smooth surfaces than on rough ones.[34] Optical phase contrast

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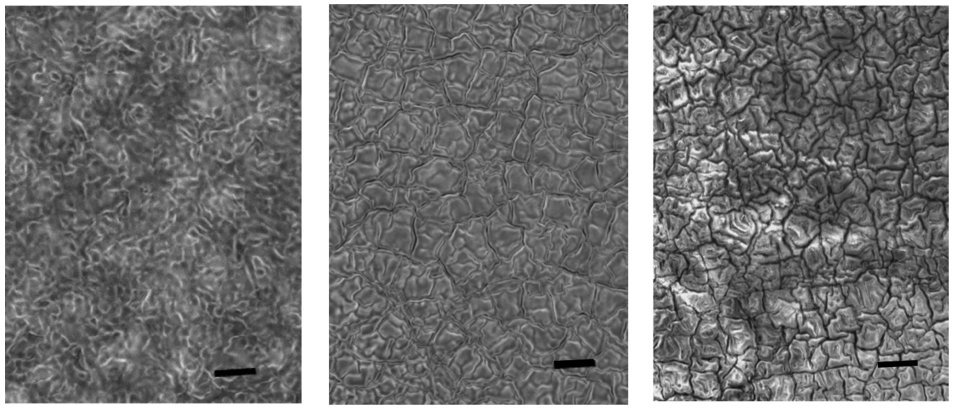

Figure 2. Phase contrast microscopy images of coated PDMS sheets. (Left panel) Pristine PDMS, (middle panel) after PEI treatment, and (right panel) PDMS after the alkylation of PEI using a proton sponge. The scale bar is 10 μm.

microscopic images (Figure 2) of the surfaces of pristine PDMS (left panel), covered with HBP and PEI (middle panel) and after alkylation in the presence of a proton sponge (Figure 2, right panel), show that the structural features of pristine PDMS are retained after applying the coating components but became somewhat more pronounced.

PEI was, for comparison, fixed directly on the coupling agent, omitting the HBPs. The Petrifilm assay showed that this coating had no bacterial growth. However, the hydrolytic stability of this coating was low. The HBP layer was hydrophobic, which prevented or at least reduced the diffusion of moisture to the interface of the coating and substrate avoiding here with the hydrolysis of the interfacial siloxy bonds. When the coating with the hydrophobic hyperbranched polymer layer was immersed for 48 h in a PBS solution at 37 °C, no reduction of the biocidal activity was observed.

Charge Density. The charge density at the interface is the most important parameter for contact killing and should surpass a threshold of approximately 10^{15} N^+/cm^2 to be effective.[35] Immobilized PEI was therefore first alkylated by bromohexane and subsequently with iodomethane, a stronger alkylating agent, to increase the charge density further.[8] The presence of positively charged nitrogen species was measured by XPS as well as with the fluorescein method.[35] XPS measures not only the fraction of nitrogen atoms in the top (10 nm) layer (N 1s electron binding energy of 399.0 eV) but also the percentage of tetraalkylated nitrogen atoms (401.4 eV, Figure S2). In a previous study, we showed an effective contact-killing coating, with the top layer containing 17 atom % nitrogen, of which 6 atom % was assigned as tetraalkylated ammonium compounds. The XPS results showed that in the top layer approximately 1 atom % of the atoms are positively charged nitrogen.[26]

The charge density as measured by the fluorescein method determines the number of positively charged nitrogen atoms per cm^2. This was calculated from the amount of fluorescein which interacts with positively charged nitrogen atoms. The amount of fluorescein absorbed was measured using UV spectroscopy (Table 1).[16]

The charge density of the PEI^+ coating was well above 10^{15} N^+/cm^2, the threshold for contact killing.[19] The fluorescein method is conducted in an aqueous environment in which most of the amino groups are protonated and identified as QACs. XPS, in contrast, is performed under high vacuum, and under these conditions, all water is removed. As a result, all amino groups will be deprotonated. Thus, XPS measures only tetra-alkylated ammonium groups, while the fluorescein method measures the alkylated and protonated QACs. Hence, the obtained charge values by the fluorescein method are an indistinguishable combination of alkylated and protonated amino groups. The fluorescein method is more representative here than XPS for measuring the charge density as bacteria proliferate only under wet conditions.

Table 1. Amount of N^+ per cm^2 of Pristine and Coated PDMS Surfaces Measured by the Fluorescein Method

sample	N^+ atoms per cm^2
pristine PDMS	0
PDMS−PEI^+ sample	$(6.8 \pm 1.2) \times 10^{16}$

Contact-Killing Efficacies. Bacterial contact killing was measured using the Petrifilm assay, applying different numbers of bacterial CFUs (colony forming units) per unit area. CFUs of >300 per cm^2 were considered to be too many to count (TMTC).

Contact-killing efficacies are summarized in Table 2 for the Gram-positive bacteria and in Table 3 for the Gram-negative bacteria. It can be seen that the QAC coatings on PDMS are highly effective in the contact killing of all Gram-positive bacteria (*S. epidermidis*, *S. aureus*, *S. salivarius*, and *Rothia dentocariosa*, Table 2). One of the objectives of this study was to use the coatings on devices for oral applications. Therefore, the antibacterial properties of the coated substrates were also tested, after applying a saliva conditioning film, for 48 h at 37 °C (entries 4b and 5b in Table 2). The interesting observation was that in spite of the large amount of protein present in saliva, the killing efficiency was not reduced, in contrast to results reported by others.[34] This observation suggests that the negatively charged bacteria were apparently able to displace preabsorbed proteins from the highly positively charged surface, resulting in killing.

Intuitively, one would expect that by adding 10 times more bacteria there would also be 10 times more survivors. However, from Tables 2 and 3 it can be seen that this is not the case. The larger the number of CFUs, the lower the percentage of surviving bacteria, something we noticed before.[8] One can only speculate on the reasons. If the surface contains some inactive spots, then bacteria will not be killed hereon. At higher CFU concentrations, the inactive spots are occupied and the influence of the active surface area becomes dominant.

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Table 2. Bacterial Contact Killing of Gram-Positive Bacterial Strains of Hyperbranched QAC Coatings on PDMS, Carried Out with a Petrifilm Assay[a]

		bacterial challenge CFU/sample			
no.	sample	10^2	10^3	10^4	% reduction
			S. epidermidis ATCC 12228		
1	control (PDMS)	69 ± 4	[b]	[b]	[b]
1a	HBP-PEI$^+$ coating	0	0	0	100
			S. epidermidis 308		
2	control (PDMS)	65 ± 7	[b]	[b]	
2a	HBP-PEI$^+$	0	0	4 ± 1.5	100
			S. aureus ATCC 12600		
3	control (PDMS)	101	[b]	[b]	[b]
3a	HBP-PEI$^+$ coating	7	[b]	11	99.9
			Streptococcus salivarius GB 24/9		
4	control (PDMS)	77 ± 6	[b]	[b]	[b]
4a	HBP-PEI$^+$	4 ± 2	12 ± 2	30 ± 5	99.7
4b	HBP-PEI$^+$ + SCF[c]	1 ± 1	8 ± 7	[b]	99.2
			Rothia dentocariosa GBJ 52/2B		
5	control (PDMS)	85	[b]	[b]	[b]
5a	HBP-PEI$^+$	9 ± 12	20 ± 7	33 ± 11	99.7
5b	HBP-PEI$^+$ + SCF[c]	1 ± 1	3 ± 1	[b]	99.7

[a]All experiments were performed in triplicate. [b]Not measured. [c]SCF, in the presence of a salivary conditioning film.

Table 3. Bacterial Contact Killing of Gram-Negative Strains on Hyperbranched QAC Coatings on PDMS, Carried Out with a Petrifilm Assay[a]

		bacterial challenge CFU/sample			
	sample	10^2	10^3	10^4	% reduction
			E. coli ATCC 1110		
1	control (PDMS)	63 ± 5	[b]	[b]	
1a	HBP-PEI$^+$	0	6 ± 2	43 ± 4	99.6
			E. coli Hu 734		
2	control (PDMS)	73 ± 5	[b]	[b]	
2a	HBP-PEI$^+$	12 ± 7	140 ± 16	[d]	86.0
2b	control + EDTA[b]	76 ± 8	[b]	[b]	
2c	HBP-PEI$^+$ + EDTA[b]	2 ± 2	12 ± 4	38 ± 8	99.6
			E. coli ATCC 15597		
3	control (PDMS)	65 ± 6	[b]	[b]	
3a	HBP-PEI$^+$	6 ± 3	65 ± 8	[d]	93.5
3b	control + EDTA[b]	76 ± 4	[b]	[b]	
3c	HBP-PEI$^+$ + EDTA[b]	0	3 ± 2	41 ± 5	99.6
			P. mirabilis 28 cii		
4	control (PDMS)	83 ± 4	[b]	[b]	
4a	HBP-PEI$^+$	65 ± 5	[d]	[d]	35
4b	control + EDTA[b]	82 ± 3	[b]	[b]	
4c	HBP-PEI$^+$ + EDTA[b]	17 ± 6	51 ± 7	55 ± 4	99.5
			P. mirabilis 296		
5	control (PDMS)	70 ± 5	[b]	[b]	
5a	HBP-PEI$^+$	34 ± 6	110 ± 8	[d]	[d]
5b	control + EDTA[b]	74 ± 3	[b]	[b]	
5c	HBP-PEI$^+$ + EDTA[b]	6 ± 3	27 ± 5	35 ± 8	99.7

[a]All experiments were performed in triplicate. [b]EDTA, ethylenediaminetetraacetic acid. [c]Not measured. [d]TMTC, too many to count.

We therefore consider the results with the highest challenge most reliable.

The results with Gram-negative bacteria were diverse (*E. coli* and *P. mirabilis*, Table 3). In some cases, the killing efficiency was high (*E. coli* ATCC 1110 and ATCC 15597), but not for all strains. Interestingly, we found that EDTA (ethylene diamine tetracarboxylic acid), a permeabilizer that weakens the shell wall,[36] increased the killing efficiency to the same level as for Gram-positive bacterial. EDTA is well known to form a complex with bivalent calcium and magnesium ions (cross-linkers) and replaces them with monovalent quaternary ammonium compounds. It is important to underline that EDTA itself is not biocidal.

Prosthesis. To demonstrate the applicability, we applied the coating on PDMS elements of a voice prosthesis as a representative example. When patients are suffering from throat cancer, their vocal cords are sometimes removed, and as a result, the patients are not able to speak. A voice prosthesis is

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an effective tool for restoring patients' speech. However, these PDMS devices are exposed to the harsh conditions in the esophagus where bacteria and yeasts are present. Antimicrobial coatings could offer a solution for prolonging the lifetime of these devices (now on average 3 months). In the absence of bacteria, yeasts may behave differently.[37,38] So far, the applicability of the antibacterial coating was investigated on flat PDMS sheets with a spin-coating procedure, which is not applicable on irregularly shaped devices. The spin-coating procedure of a semispherical object was not feasible and was replaced by a dip-coating process. The concentration of the PEI solution was varied (5, 10, and 20 wt %) to determine the influence of the number of amino groups on the surface. After alkylation with bromohexane and iodomethane, the coated elements were immersed in a suspension of 3×10^8/mL of *S. epidermidis* for 3 h at 37 °C. The samples were then washed with sterile water and incubated for 48 h at 37 °C, according to the Petrifilm assay. It can be seen (Table 4) that when the PEI concentration was >10 wt %, resulting in a high charge density, good biocidal behavior was obtained with dip-coated semispherical objects with *S. epidermidis* ATCC 12228.

Table 4. Antibacterial Results of Semispherical Parts of the Voice Prosthesis with *S. epidermidis* ATCC 12228[a]

sample	number of bacteria
blank	TMTC[b]
5% PEI	TMTC[b]
10% PEI	0
20% PEI	few dots

[a]The concentration of PEI in methanol is varied. [b]TMTC, too many to count.

CONCLUSIONS

The antibacterial efficacy of PDMS substrates coated with an immobilized antibacterial hyperbranched coating provided with quaternary ammonium compounds was evaluated for five Gram-positive and five Gram-negative bacteria by using a Petrifilm assay. The hyperbranched coated resins were successfully immobilized, as mediated by a coupling agent. The coupling agent comprised siloxane moieties, to couple onto the silanol groups of plasma-treated PDMS, and blocked isocyanates to react with the secondary amino groups of the hyperbranched polymers. Advantage was taken of the selectivity of BIs to react with amines and not with surface silanol groups. The tethered hyperbranched polymers were provided with amino groups by immobilizing polyethylenimine on the surface via the BIs located at the end of each branch of the HBPs. The cross-link density of the coating was controlled by the reaction time with PEI. Firm adhesion was demonstrated because an extraction with DMF overnight at 115 °C did not remove the coating. After alkylation, the charge density was above the threshold (>10^{15} N^+/cm^2 by the fluorescein method) and was effective. The coatings appeared to be highly efficient in killing Gram-positive bacteria and showed sufficient results with Gram-negative bacteria. Some bacterial strains were killed efficiently and some were killed less efficiently. However, in the presence of a permeabilizer (EDTA) the coatings became equally effective for Gram-negative as for Gram-positive bacteria. Importantly, the activity of the coatings was not influenced by the proteins of saliva condition films.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.9b02549.

Alkylation with hexyl bromide in the presence of KOH or with a proton sponge and the nitrogen XPS spectrum of antibacterial hyperbranched coating on PDMS (PDF)

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Notes

The authors declare no competing financial interest.

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